AD_____

Award Number: W81XWH-09-1-0436

TITLE: Vascularizing Bone Scaffolds for Craniofacial Reconstruction

PRINCIPAL INVESTIGATOR: Dr. Hyun-Duck Nah-Cederquist

CONTRACTING ORGANIZATION: Children's Hospital of Philadelphia
Philadelphia, PA 19104

REPORT DATE: July 2011

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2011	Final	15 JUN 2010 - 14 JUN 2011

4. TITLE AND SUBTITLE

Vascularizing Bone Scaffolds for Craniofacial Reconstruction

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0436

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Dr. Hyun-Duck Nah-Cederquist

E-Mail: nah@email.chop.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Children's Hospital of Philadelphia
Philadelphia, PA 19104

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Abstract on next page.

15. SUBJECT TERMS

Subject terms on next page.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	26	**19b. TELEPHONE NUMBER** *(include area code)*

14. ABSTRACT

This project addresses a deployment-related research gap, 'Biocompatible craniofacial implant for use in craniofacial reconstruction due to combat trauma' within an FY08 DRMRP topic area. Extensive injuries to the head and face require immediate and follow-up reconstructive surgeries to restore skeletal structures. Current surgical procedures commonly involve the use of autografts, allografts or polymeric and metallic implants, which fall short of the ideal with numerous limitations. An ideal bone substitute would be a biocompatible and biodegradable material that can serve as a transient scaffold while guiding and stimulating host bone tissue regeneration. While impressive progress has been made particularly in material sciences and fabrication technologies, clinical application of bone tissue engineering remains elusive, particularly to repair large defects. This limitation can be traced largely to a difficulty of introducing a stable and functional vascular network into engineered constructs. Thus, the goal of this project was to develop a cell and growth factor-based regenerative tissue engineering approach to the repair of a large bony defect. In this study, we demonstrate that inclusion of both angiogenic and osteogenic cells was critically important to ensure rapid vascularization of the bone construct. We also prepared and characterized biodegradable PLGA microspheres that release angiogenic and osteogenic factors in a temporally controlled manner. These microspheres were highly effective for supporting survival and differentiation of co-implanted cells in bone scaffolds implanted in animals.

15. SUBJECT TERMS

Biocompatible Craniofacial Implant for Use in Craniofacial Reconstruction due to Combat Trauma, Vascularization, Scaffold-Guided Regenerative Bone Tissue Engineering, Mesenchymal Stem Cells, Controlled Delivery of Growth Factors

Table of Contents

	Page
Introduction……	1
Body……	1-8
Key Research Accomplishments……	8
Reportable Outcomes……	9
Conclusion……	9-10
References……	10
Appendices……	10-12
Supporting Data ……	13-22

A. INTRODUCTION:

Severe impact injuries to the head and face from combat trauma result in large defects in the skull, requiring reconstructive surgeries to rebuild lost bony structures. To date, the quest for a viable and readily available bone substitutes to restore large defects continues to elude surgeons. The repair of large defects remains to be the major challenge due to difficulties in inducing vascular networks in the grafts. Bone is a highly vascularized tissue, and its viability critically depends on nutrients and oxygen delivered by blood vessels. Angiogenesis is therefore an essential pre-requisite for 3-D scaffold-guided reconstruction of large bone defects. A host of bioactive molecules have been identified to regulate angiogenesis and osteogenesis. Particularly, the combined FGF2 and VEGF treatment showed superior effect on neovascularization [1]. Similarly, bone formation induced by sequential treatment with FGF2 followed by BMP2 was shown to be much more extensive than by a single growth factor treatment [2] Therefore, controlled delivery of osteogenic and angiogenic growth factors and cells from biocompatible scaffolds to repair sites may prove to be a new and significant therapeutic solution for a large bone defect. The objective of this project is to develop a multiple cell- and growth factor-based approach to induce rapid neovascularization in 3-D scaffold-guided bone tissue regeneration.

B. BODY:

Specific Aim 1. To construct a 3-D printed PLGA/PCL-TCP scaffold that will allow temporally controlled release of multiple angiogenic and osteogenic growth factors.

In ***Task 1-1,*** we proposed to prepare biodegradable polymeric microspheres that will allow early release of FGF2 and VEGF and delayed and sustained release of BMP2.

A variety of techniques have been described in the literature regarding the fabrication of microspheres. The most widely used approach has been a double emulsion extraction technique. With this method, homogenizing equipment or ultrasonic probes are used to create an aqueous emulsion of growth factors and polymer (i.e. the materials to construct the microsphere), suspended in a separate solvent that is not miscible with water. This technique, while reliable for producing microspheres, does not allow for precise control over microsphere size, which could be critical for achieving the controlled release of growth factors.

During the first year of the study, we tested an inkjet-based technology combined with

single emulsion-solvent evaporation techniques developed by MicroFab to produce individual microspheres with predetermined sizes. This approach employs an inkjet-based nozzle to control the size of individual microspheres, ejecting solutions of polymer and growth factor through a pre-determined nozzle size into a dispersant medium as a technique for capturing individual microspheres. While MicroFab Lab has successfully used this approach to load Taxol in PLGA microspheres, encapsulation of protein growth factors has not been tested.

In collaboration with MicroFab Lab, FGF2 encapsulation was tried. Briefly, FGF2 solution was emulsified in dichloromethane containing PLGA (50:50), dispensed into dichloromethane through a nozzele to obtain microspheres of 100 μM in diameter. Before starting the fabrication of the microspheres, we had confirmed in cell culture studies that FGF2 did not lose bioactivity after 24-72 hour incubation in dichloromethane. The microspheres loaded with FGF2 were found to fall within a 50-150 micron size range (**Fig 1 and 2**). Subsequently, FGF release from microspheres was evaluated for by using an enzyme-linked immunosorbent assay (ELISA); the loaded microspheres were incubated for 2 weeks in culture media and aliquots of conditioned media were collected daily and assayed using an ELISA kit (FGF2 quantification R&D). The results showed that FGF2 was not detectable in the media for the duration of the first 2 weeks (data not shown). FGF2 is a highly charged molecule. Due to its chemical properties, FGF2 might have leached out from microspheres into a dispersant medium during fabrication. Alternatively, FGF2 in PLGA spheres might not have released during the first two weeks.

During the second year of the study, we tested one of the emulsion solvent extraction–evaporation methods for protein encapsulation. Specifically, we used the double emulsion water-in-oil-in-water (W/O/W) technique to fabricate growth factors and BSA containing PLGA microspheres. Briefly, proteins were dissolved in a 50 μl PBS solution (internal water phase), which was then emulsified with 1 ml PLGA/dimethyl chloride solution (oil phase) using a vortex. The resulting first emulsion was vortexed into 250 ml 70% PVA prepared with PBS (pH7.4) (external water phase) to produce the double W/O/W emulsion. It was stirred for an additional 30 min until microspheres were hardened. Subsequently, dimethylene chloride was removed from the internal phase by adding additional 750 ml 70% PVA prepared in PBS to the double emulsion solution. The resulting microspheres were washed in milli-Q water three times, freeze-dried and stored at -80°C.

Protein release from PLGA microspheres depends on their degradation time, which can be altered by the molecular weight and concentration of polymers, the ratio of lactic vs glycolic acid in the polymer, and the structure of the microspheres. To define the microsphere fabrication conditions for FGF2, VEGF and BMP2, we first determined the influence of molecular weight and concentration of PLGA on protein encapsulation efficiency, the protein release kinetics and the structure of microspheres. PLGA with three different molecular weights (Low, 7-17kDa (75:25); Medium, 38-54kDa (50:50); High, 66-117kDa (75:25)) were fabricated in three different concentrations (10, 15 and 20%). For these microspheres, BSA was encapsulated. The yield of harvested microspheres was generally high, ranging from 76-94% (**Table 1**). Protein encapsulation efficiency varied largely depending on the concentration of polymers. 20% consistently showed higher protein encapsulation than 10 or 15% regardless the molecular weight of the polymer (**Table 1**).

The morphology of the PLGA microsphere was strikingly different depending on the molecular weight and concentration of the polymers. As shown in **Fig 3**, microspheres prepared with the low molecular weight (LMW) PLGA were very fragile. Especially larger spheres were mostly broken as indicated with yellow arrows (**Fig 3, first row**). Microspheres with medium molecular weight (MMW) PLGA showed distinctly different morphology depending on the concentration of the polymer. As the concentration increases, the sizes of the microspheres also increased. Furthermore, the number of internal pores also increased (**Fig 3, second row**). The trend was consistent with the high molecular weight (HMM) PLGA, in that the microsphere size and the number of internal pores increased as the PLGA concentration increased (**Fig 3, third row**). Compared to MMW PLGA, HMW PLGA formed the larger spheres with more internal pores (**Fig 3**). To better characterize the structure of microspheres, rhodamine tagged BSA was encapsulated in MMW PLGA microspheres and images by confocal microscopy. Representative images of microspheres shown in **Fig 4** confirmed that the concentration of PLGA positively correlates with the number of protein-containing internal pores.

Microspheres described above were characterized for the protein release kinetics. For the release study, 3 mg of BSA containing HMW PLGA microspheres were placed in the upper chamber of a co-culture well and incubated in PBS at 37°C for 35 days. Aliquots of the solution were regularly removed from the bottom chamber of co-culture wells during the incubation period and assayed for protein concentration. The result shows that protein release profiles were

distinctly different among the microspheres with three different concentrations (**Fig 5A-C**). The burst rates of 10%, 15 and 20% HMW PLGA microspheres were about 10, 30 and 60%. 80% of encapsulated protein was released from 10 and 15% PLGA within the first 5 and 15 days of incubation, respectively. Nearly 100% of the encapsulated protein was released from both 10 and 15% PLGA microspheres within the first 35 days. In contrast, after the initial burst, the 20% HMW PLGA microspheres showed a lag period for the next 7-10 days, which was followed by a slow and sustained release. About 35-40% of the encapsulated protein was released from the 20% PLGA microspheres by the end of the incubation period. We are currently working on long-term release kinetics on this microspheres. Despite that there were differences in the release profiles among different concentrations of MMW PLGA microspheres, they were subtle with high burst rates. **Fig 6** shows profiles of relative protein release from three different groups.

In ***Tasks 1-2 and -3***, we prepared growth factor containing microspheres. Based on protein release kinetics determined from the various types of microspheres, we decided to use 15% MMW-PLGA for FGF2 and VEGF, and 20% HMW-PLGA for BMP2. Using Elisa, we determined that encapsulation efficiency for FGF2 and BMP2 was about 20% and 70 %, respectively. We next tested bioactivity of encapsulated growth factors. For FGF2-MS bioactivity, we used cell survival assays. Briefly, human umbilical vein endothelial cells (HUVEC) were serum-deprived (2.5%) for 3 days in the presence of FGF2 microspheres (FGF2-MS), free *rh*FGF2, or BSA-MS. FGF2-MS tested in this assay contains10 ng of FGF2 in each mg of MS. At the end of the experiement, cell survival was determined by MTT assays. We found that FGF2-MS was highly effective in keeping cells viable under a serum-deprived condition (**Fig 7**). Interestingly, FGF2 in MS was more effective than the same amount of free rhFGF2 directly added to cells once in the beginning of the experiment. This finding further justify the effectiveness of MS as a growth factor delivery vehicle for a protracted period. BSA-MS treated cells showed no difference from the untreated cells, confirming that FGF2 encapsulated in MS maintained its bioactivity.

We also tested bioactivity of BMP2 encapsulated in the MS. For this test, we treated MC3T3 osteoblast like cells with BMP2-MS and free rhBMP2 for 3 days and examined for the induction of alkaline phosphatase activity. For this short-term assay, we used 10% HMW PLGA MS containing BMP2. As shown in **Fig 8**, BMP2-MS induced alkaline phosphatase protein expression and activity, confirming that BMP2 encapsulated in the PLGA MS is bioactive.

Specific Aim 2. To evaluate angiogenic and osteogenic effects of the FGF2/VEGF/BMP2 loaded and cell seeded PLGA/PCL-TCP scaffold constructs in vivo.

In this Aim, we tested the *in vivo* potential for growth factors- and cell-loaded scaffolds. As stated above, while working on modifications for the fabrication process of growth factor loaded microspheres, we had initiated *in vivo* studies (*Study 1* and *Study 2*), in which we tested cell types needed for vascularized engineered bone construct. Following the successful encapsulation of growth factor containing PLGA microspheres, these microsphere were tested for their bioactivity *in vivo* (*Study 3*)

Study 1 - We first determined that seeding a large scaffold with endothelial cells (HUVEC) together with osteogenic cells (BMP2 treated bone marrow-derived mesenchymal stem cells (BM-MSCs)) ensures vascularization and survival of engineered bone constructs. For this study, the porous scaffolds were freeform fabricated from a mix of 30% PCL and 70% beta-TCP (Therics) to a dimension of 6x6x6 mm with 70% porosity. All scaffolds were sterilized using ethelyene oxide gas for 20 hours at 37C. Bone marrow derived mesenchymal stem cells (BM-MSCs) and hematopoietic stem cells (HSCs) from healthy donors were obtained from the University of Pennsylvania Stem Cell Core Facility. BM-MSCs were expanded using DMEM supplemented with 10% FBS and 1% antibiotics. HUVECs were purchased from AllCells and cultured in HUVEC supplemental medium. Passage 5 cells were used for seeding scaffolds. $6x10^5$ cells (MSC+HUVEC, MSC+HSC, or MSC alone) in 4U/mL of thrombin was loaded in each scaffold and sealed with 20mg/mL of fibrinogen. The cell seeded-scaffolds were cultured overnight to ensure cell attachment before implanting into dorsal subcutaneous pockets prepared in a 6 week-old athymic nude mouse. After 8 weeks, the mice were systemically perfused with a silicone-based vascular contrast agent (Microfil) by intra-cardiac injections for micro-CT analyses. The bone scaffolds were harvested, fixed and decalcified.

Micro-CT analysis of and vascular volumetric plotting for harvested implants demonstrated the formation of substantial amounts of microvascular beds in the MSC+HUVEC constructs; whereas MSC alone, MSC-HSC, and cell-free constructs demonstrated markedly less neovasculature (**Fig 9**). Volumetric analysis showed that MSC-HUVEC constructs contained 250% more microvasculature than cell-free construct and 180% more microvasculature than MSC or MSC-HSC constructs. Additionally, neovascularization in the cell free construct was mostly limited to the surface of the construct. These findings demonstrate that angiogenic cell

component may be needed for engineering of a viable large bone construct. Histology of the constructs confirms micro-CT data (**Fig 9**)

Study 2 - Previous studies have shown that UCB-MSCs are capable of differentiating into multiple lineages *in vitro* [6]. Therefore, UCB-MSCs may be differentiated into both osteogenic and angiogenic cells if they are exposed to right growth factor treatment conditions. In addition, these cells have a higher proliferative capacity than their non-umbilical cord blood-derived counterparts, offering UCB-MSCs as an ideal cell source for cell-based bone tissue engineering for large defect repairs. However, little is known about the growth factor treatment conditions for angio- and osteogenic differentiation as well as their ability to form *de novo* microvasculature and bone tissue when implanted with scaffolds.

In this study, we hypothesized that a sequential treatment of UCB-MSCs with FGF2 followed by BMP2 would induce osteogenic differentiation, while a sequential treatment of the cells with FGF2 followed by VEGF would induce angiogenic differentiation. To test this hypothesis, UCB-MSCs were sequentially treatment with 5 ng/ml FGF2 for one week followed by 50 ng/ml BMP2 for an additional two/three weeks to induce osteogenic differentiation, and examined for alkaline phosphatase activity and mineral deposition. As shown in **Fig 10A** & **B**, sequential FGF2 and BMP2 treatment significantly increased alkaline phosphatase positive cells. For angiogenic differentiation, UCB-MSCs were treated with 5 ng/ml FGF2 followed by 20 ng/ml VEGF for two weeks and analyzed for capillary tubule formation by Matrigel assays. As shown in **Fig 10C** & **D**, the cells treated with FGF2 and VEGF formed capillary tubules (**Fig 10D**) similar to differentiated endothelial cells, HUVEC (data not shown). We plan to perform Flow cytometric analysis with the UCB-MSCs grown under the angiogenic condition for cell surface markers to further characterize the proportion of angiogenic cells in the FGF2-VEGF treated cells.

To determine the ability of UCB-MSCs to form microvasculature and bone *in vivo*, UCB-MSCs were pretreated with growth factors as described above and prepared a large number of angiogenic and osteogenic UCB-MSCs. PCL-βTCP scaffolds were then seeded with predifferentiated UCB-MSCs in $6x10^5$ cells/scaffold (3 Groups: angio+osteo cells, angio cells alone, no cells), cultured overnight and implanted in dorsal subcutaneous pockets of athymic nude mice. In order to track cell fate after implantation, UCB-MSCs were transduced with a

lentiviral construct containing genes for both green fluorescent protein (GFP) and luciferase before treating with growth factors. Post-implantation cell survival/growth/migration were assessed weekly, using a non-invasive *in vivo* imaging technology (IVES) for the implanted cells with luminescent labeling. At the end of the 8-week implantation period, mice were perfused with Microfil, a radioopaque contrast dye, and the harvested scaffolds were analyzed by microCT and histology.

In vivo imaging data showed that UCB-MSCs seeded in 3-D scaffolds were viable for the duration of the experimental period and stayed largely at the site of implantation (**Fig 11A**). However, it was noted that a small portion of the labeled cell population migrated afar into other highly vascularized areas, such as footpad and liver (data not shown). One interesting possibility is that UCB-MSCs may have entered into circulation and homed at the site of injuries or ischemia. We are following up on this interesting observation to further characterize the fate of implanted cells. Histological analysis of implants harvested from nude mice demonstrated the formation of substantial amounts of microvascular beds in the scaffold seeded with angiogenic and osteogenic UCB-MSCs (**Fig 11D**) and angiogenic UCB-MSCs (**Fig 11C**). There was more microvasculature in the scaffolds seeded with both angio- and osteogenic cells compared to those with angiogenic cell alone. Meanwhile cell-free scaffolds showed no microvasculature (**Fig 11B**).

Study 3 – In this study, the effect of FGF2-MS on angiogenic cell survival and differentiation was tested *in vivo*. When angiogenic cells were co-implanted with scaffolds, significant number of cells do not survive and seem undergo apoptosis *in vivo*. Thus we studied cell survival and angiogenic effect of FGF2-MS *in vivo*. For this study, 200 ul matrigel containing HUVEC only, FGF2-MS only, or HUVEC plus FGF2-MS, was subcutaneously injected. The injected gel was solidified within 30 min. The matrigel scaffold was harvested in 10 days and examined by histology. The gel only group showed neither cells nor blood vessels (**Fig 12A**). The HUVEC only group showed only a small number of surviving cells scattered within the gel (**Fig 12B**). On the other hand, the group with both HUVEC and FGF2-MS showed numerous cells not only surviving but also forming blood vessels (**Fig 12C**). Interestingly, the FGF2-MS only group also showed presence of cells in vicinity of microspheres (**Fig 12D**). These cells are host cells migrating into the gel. Blood vessel formation was not observed in the FGF2-

MS only group. Our future studies will determine the most effective combination of growth factor containing microspheres and cells towards a vascualrized bone construct.

C. KEY RESEARCH ACCOMPLISHMENTS:

1. ***We showed that a large PCL-βTCP scaffold seeded with HUVEC together with BM-MSCs become highly vasculaized in vivo.*** The scaffold seeded with BM-MSCs alone failed to form a vascular network within the scaffold. This proof of principle study underscores the importance of including an angiogenic component in the preparation of a large bony construct.

2. ***We determined the growth factor treatment conditions that allow both cell expansion and differentiation of UCB-MSC into angio- and osteogenic cell lineages***. Pretreatment of UCB-MSCs with FGF2 allowed cell growth without compromising either of their osteo- or angiogenic potential. Following the FGF2 treatment, a sequential treatment with VEGF reliably induced angiogenic differentiation, and with BMP2 osteogenic differentiation, from a single cell source UCB-MSC. These conditions were optimized and tested for their maximum efficacy, using various in vitro assays. These combination growth factor treatments allows the preparation of sufficient amounts of both angio- and osteogenic cells, which is necessary for cell-based bone tissue engineering.

3. ***We demonstrated that a large PCL-βTCP scaffold seeded with predifferentiated angio- and osteogenic UCB-MSCs become highly vascularized in vivo.*** We also showed that the vascular network formation was much more robust when angiogenic cells were co-implanted with osteogenic cells. The microvasculature formed from the predifferentiated UCB-MSCs was contiguous with systemic circulation and remain stable for a prolonged period.

4. ***We successfully modified protein encapsulation conditions to achieve temporally controlled protein release.*** We used the double emulsion water-in-oil-in-water (W/O/W) technique for protein encapsulation. By changing the molecular weights and concentration of polymers, we were able to control the encapsulated protein release kinetics from microsphere.

5. ***We successfully encapsulated FGF2, VEGF and BMP2, and tested for their bioactivity.*** FGF2-MS drastically enhanced the survival of angiogenic cells and vasculogenesis *in vivo*. BMP2-MS also showed bioactivity, inducing alkaline phosphatase activity in a osteoblast-like cell line.

D. REPORTABLE OUTCOMES:

1. Hindin,D, Stosich, M., Salhab, I., Yang, B.-H., Serletti, J.M., Nah, H.-D. Thrombin biomatrix and HUVEC/HSC enhance vascularization of a large PCL-βTCP bone construct implanted in nude mice. Presented at the 89th Annual Meeting of the American Association of Plastic Surgeons in San Antonio, TX, March 20-23, 2010.

2. David Hindin, Michael Stosich, Jason Laurita, Hyun-Duck Nah. Multipotent Umbilical Cord Blood-Derived MSCs Induce Neovascularization of a Large PCL-Beta TCP Bone Construct Implanted in Nude Mice. Accepted for Presentation at the 27th Annual Meeting of Northeastern Society of Plastic Surgeons in Washington, DC, October 28-31, 2010

E. CONCLUSION:

In this study, we demonstrated that inclusion of both angiogenic and osteogenic cell components maybe necessary to ensure viability of engineered bone by rapidly introducing the extensive network of microvasculature to the construct. We have also determined that both angiogenic and osteogenic differentiation can be readily induced from a single cell source, UCB-MSCs, by combination treatments with FGF2/VEGF/BMP2. Cord blood-derived cell populations are particularly attractive for bone tissue engineering because they not only possess pleuripotency but also can be easily expanded in culture to prepare a sufficient number of cells needed for a large defect repair. In addition, we prepared FGF2 and VEGF containing 15% MMW PLGA microspheres for early release and BMP2 containing 20% HMW PLGA microspheres for sustained release. Co-implanting FGF2-containing PLGA microspheres was highly effective for the survival of implanted cells and endotheial cell differentiation. We have duplicated or triplicated most of our experiments presented in this report. As we complete data analyses, we will prepare a manuscript for publication.

Scaffold-guided bone tissue engineering has been the one of the most studied topics in the field of tissue engineering. While impressive progress has been made particularly in material sciences and fabrication technologies, clinical application of bone tissue engineering remains elusive, particularly to repair large defects. This limitation can be traced largely to a difficulty of

introducing a stable and functional vascular network into engineered constructs. The overall outcome of this study is intended to advance our ability to overcome such limitation.

F. REFERENCES:

1. S. T. Nillesen *et al.*, *Biomaterials* **28**, 1123 (Feb, 2007).
2. A. Fakhry *et al.*, *Bone* **36**, 254 (Feb, 2005).
3. X. H. Zhu, C. H. Wang, Y. W. Tong, *J Biomed Mater Res A* **89**, 411 (May, 2009).
4. P. Yilgor, N. Hasirci, V. Hasirci, *J Biomed Mater Res A* **93**, 528 (May).
5. B. Duan, M. Wang, *J R Soc Interface*, (May 26).
6. V. Markov *et al.*, *Stem Cells Dev* **16**, 53 (Feb, 2007).

G. APPENDICES: 12

Abstract 1: *Presented at the 2010 AAPS Annual Meeting*

Thrombin Biomatrix and HUVEC/HSC Enhance Vascularization of a Large PCL-βTCP Bone Construct Implanted in Nude Mice

David Hindin, B.A.[1,2], Michael Stosich, D.M.D., M.S.[2], Imad Salhab, M.S.[2], Byung-Ho Yang, D.D.S., Ph.D.[2], Josef M. Serletti[1], Hyun-Duck Nah, D.M.D., Ph.D.[1,2]. The University of Pennsylvania School of Medicine; Division of Plastic and Reconstructive Surgery[1], The Children's Hospital of Philadelphia, Philadelphia, PA, USA, Division of Plastic and Reconstructive Surgery[2], Philadelphia, PA, USA.

Purpose - In the scaffold-guided repair of large, voluminous bony defects for craniofacial reconstruction, the creation of vascular networks is critical to the success of subsequent bone formation within the scaffold. Thrombin has been suggested to play a role in the initiation, regulation, and organization of angiogenic molecules. In this study, we assessed the angiogenic effect of thrombin by determining its ability to induce VEGF expression in human umbilical vein-derived endothelial cells (HUVEC) and osteogenic mesenchymal stem cells (MSCs). We then asked whether co-implantation of HUVECs or hematopoietic stem cells (HSCs) with osteogenic MSCs would enhance the *in vivo* formation of microvascular networks in a large bone construct.

Methods - In vitro studies were performed by exposing HUVECs and MSCs in culture to various concentrations of thrombin (0, 0.5, 1 and 2 U/mL). VEGF gene expression was assessed using RT-qPCR. For *in vivo* studies, a hybrid scaffold of TCP/PCL (30%TCP:70% PCL; 6x6x6 mm; 65% porosity) with interconnected open pores was 3-D printed to serve as an osteoinductive matrix. Groups (N=4) consisted of scaffold samples seeded with MSCs, MSCs-HUVECs, MSCs-HSCs, and no cells. To promote vasculogenesis, cells were suspended in thrombin and seeded into the microporous constructs, sealed with fibrinogen, and subcutaneously implanted into immunodeficient athymic nude mice. At 8 weeks, mouse blood vessels were perfused with

radiopaque microfil. The scaffolds were harvested, demineralized and analyzed for neovasculature by microcomputed tomography (micro-CT).

Results – Thrombin increased VEGF gene expression by several-fold in HUVECs. This effect was specific for HUVECs and was not observed in MSCs. Implants harvested from nude mice demonstrated the formation of substantial amounts of microvascular beds in the MSC-HUVEC constructs as illustrated by micro-CT and vascular volumetric plotting; MSC, MSC-HSC, and cell-free constructs demonstrated markedly less neovasculature. Volumetric analysis showed that MSC-HUVEC constructs contained 250% more microvasculature than cell-free constructs, and 180% more microvasculature than MSC or MSC-HSC constructs. Additionally, neovascularization in the cell-free construct was limited to the surface of the construct.

Conclusion – The findings from this study demonstrate that the use of a thrombin biomatrix and the addition of HUVEC cells drastically enhanced microvascular network formation in large bone constructs. Thrombin induced upregulation of VEGF in HUVECs, suggesting an explanation for its angiogenic influences when utilized within the scaffold. The development of neovascularization from noninvasive sources constitutes an important step in the advancement of clinical strategies for tissue vascularization of large craniofacial defects.

Abstract 2: ***Accepted for Presentation at the 2010 NESPS Annual Meeting***

Multipotent Umbilical Cord Blood-Derived MSCs Induce Neovascularization of a Large PCL-Beta TCP Bone Construct Implanted in Nude Mice

David Hindin[1,2], Michael Stosich[2], Jason Laurita[2], Hyun-Duck Nah[1,2]. University of Pennsylvania, Philadelphia, PA, USA[1], Children's Hospital of Philadelphia, Philadelphia, PA, USA[2].

Background - In the scaffold-guided repair of large, voluminous bony defects for craniofacial reconstruction, the creation of vascular networks is critical to the success of subsequent bone formation within the scaffold. Research has shown that a combination of osteogenic and angiogenic cells, such as human umbilical vein-derived endothelial cells and bone marrow mesenchymal stem cells, are critical to stable scaffold microvasculature formation. In order to ultimately translate tissue-engineered microvasculature to clinical use however, technical challenges such as immune matching have created an impetus to achieve microvasculature formation from a single cell source. In this study we evaluated the potential of a single cell type, umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs), to undergo both osteogenic and angiogenic differentiation. We then asked whether implantation of UCB-MSCs in a large bone construct would lead to *in vivo* microvascular network formation in nude mice.

Methods - *In vitro* studies were performed by treating UCB-MSCs with either osteogenic or angiogenic conditions. Osteogenic conditions were established by supplementing media with 100nM dexamethasone, 0.05mM ascorbic acid, and 10ng/mL fibroblast growth factor (FGF). Angiogenic conditions consisted of media supplemented with 10ng/mL FGF and 100ng/mL vascular endothelial growth factor (VEGF). Osteogenic differentiation was assessed by evaluating alkaline phosphatase staining after five days. Angiogenic differentiation was assessed using an *in vitro* Matrigel angiogenesis assay. For *in vivo* studies, a hybrid scaffold of TCP/PCL (30%TCP:70% PCL; 6x6x6 mm; 65% porosity) with interconnected pores was 3-D printed to serve as an osteoinductive matrix. Groups (N=4) consisted of scaffolds seeded with angiogenic-

treated UCB-MSCs, angiogenic-treated and osteogenic-treated UCB-MSCs, and no cells. At 4 weeks, scaffolds were harvested, demineralized, and prepared for histological analysis.

Results - UCB-MSCs treated with osteogenic conditions *in vitro* demonstrated increased alkaline phosphatase staining compared with non-treated UCB-MSCs. In addition, UCB-MSCs that underwent *in vitro* angiogenic treatment demonstrated significant capillary tube formation on Matrigel, a finding not seen with untreated UCB-MSCs. Histological analysis of implants harvested from nude mice demonstrated the formation of substantial amounts of microvascular beds in the scaffold seeded with angiogenic UCB-MSCs and the scaffolds seeded with angiogenic and osteogenic UCB-MSCs, while cell-free scaffolds showed no microvasculature.
Conclusions - The formation of microvasculature represents a crucial element necessary for the ultimate success of any scaffold-guided approach to bone tissue regeneration. The findings in this study demonstrate that UCB-MSCs can be readily differentiated into both osteogenic and angiogenic cell types that are capable of inducing microvascular formation in large bone constructs. This represents an important step in the advancement of clinical strategies for tissue vascularization of large craniofacial defects.

H. SUPPORTING DATA

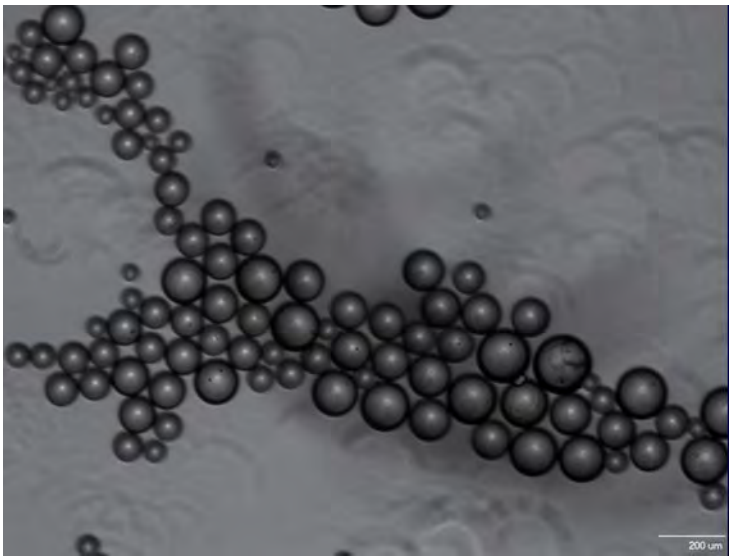

Figure 1. PLGA microspheres loaded with FGF2. They were prepared using an inkjet-based technology combined with single emulsion-solvent evaporation techniques, developed by MicroFab

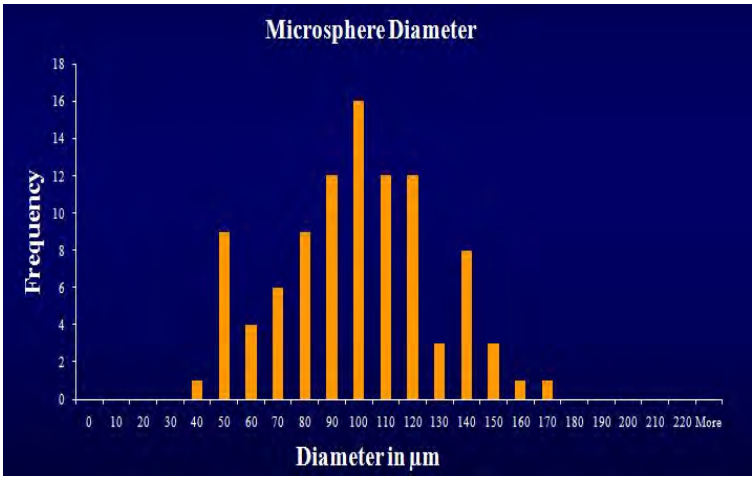

Figure 2. Size distribution of FGF2 loaded PLGA microspheres prepared by MicroFab

Table 1. Summary of yield, protein encapsulation efficiency and protein content of the PLGA microspheres prepared by double emulsion

PLGA		Yield (%)	Encap. Effi. (%)	Protein/Microspheres (μg/mg)
MW (kDa)	*Conc. (%)			
HMW, (66-107)	**10**	83.5	66.1	58.6
	15	87.7	67.7	43.3
	20	85.4	76.4	37.6
MMW, (38-54)	**10**	91.4	64.1	57.4
	15	94.3	81.3	50.5
	20	91.5	88.1	41.7
LMW, (7-17)	**10**	76.1	18.70	30.6
	15	79.7	63.50	73.8
	20	83.6	78.90	70.8

* Concentration (Conc.) is determined by PLGA/DCM (w/v).

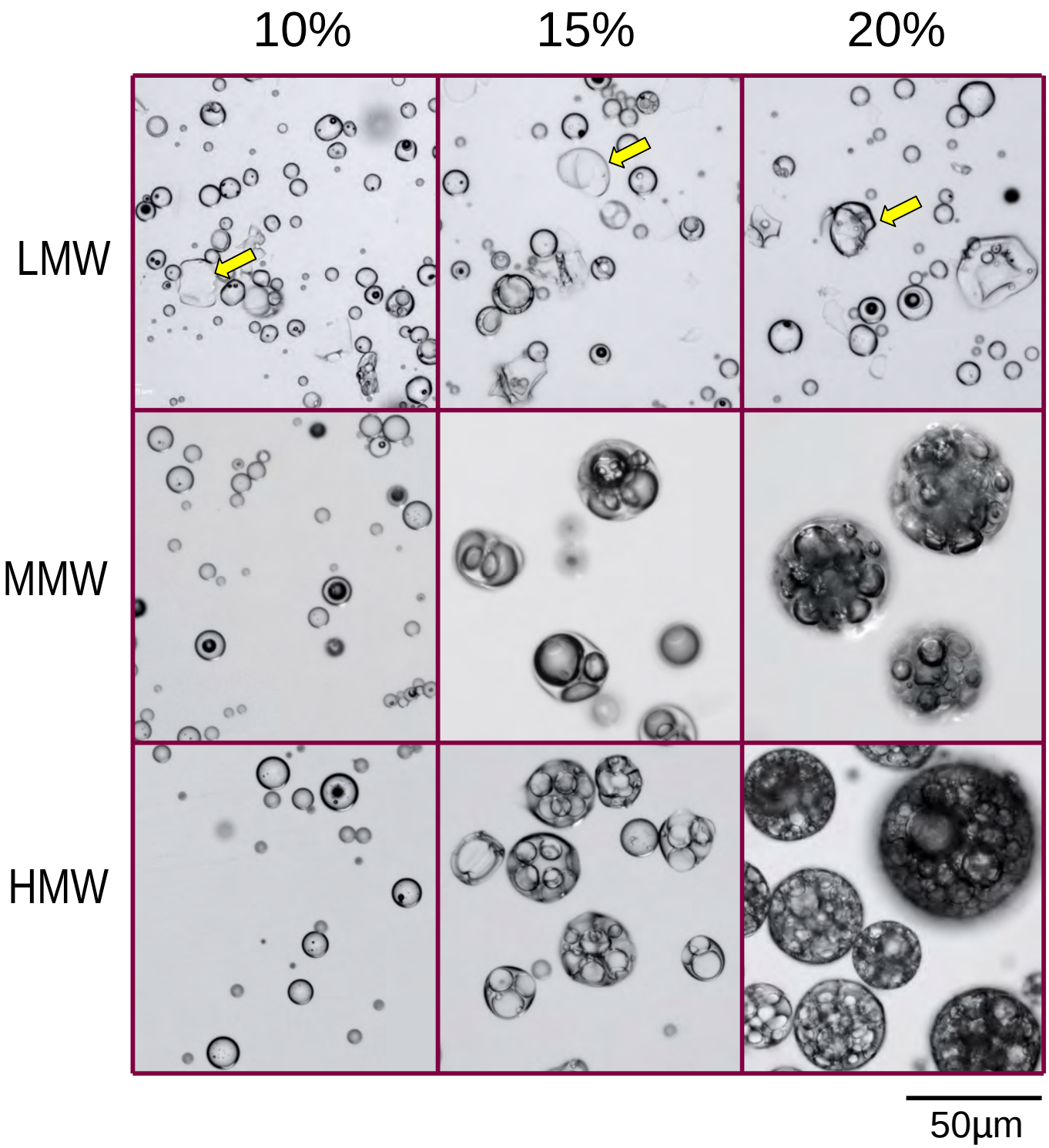

Figure 3. BSA loaded PLGA microspheres prepared with low molecular weight (LMW, 75:25), medium molecular weight (MMW, 50:50) and high molecular weight (HMW, 75:25) polymers at the concentrations of 10, 15 and 20%. Arrows points to broken microspheres prepared with LMW polymers.

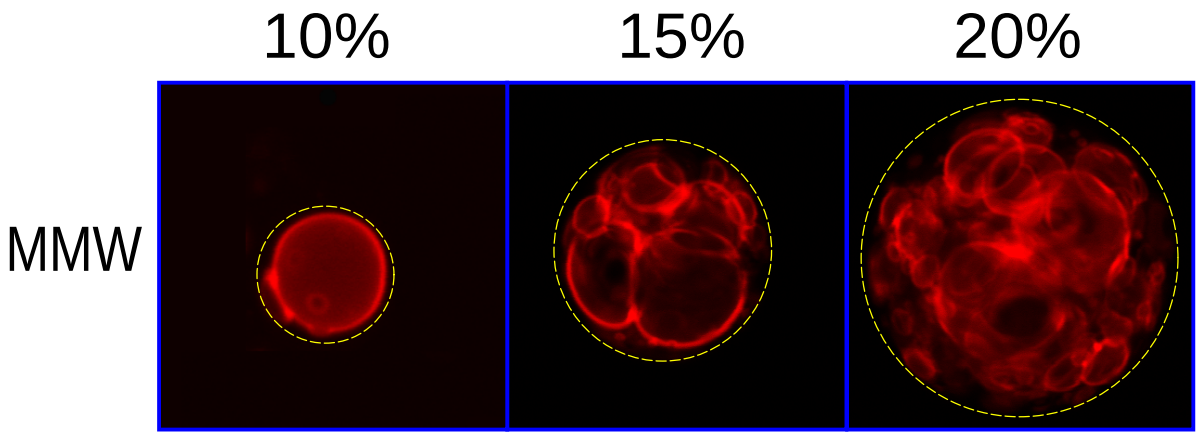

Figure 4. Confocal microscopy of rhodamine labelled BSA-loaded MMW PLGA microspheres (MS). MS prepared with MMW 10% PLGA shows one large internal pore. The size of MS and the number of internal pores in the MS increase as the concentration of polymer increases.

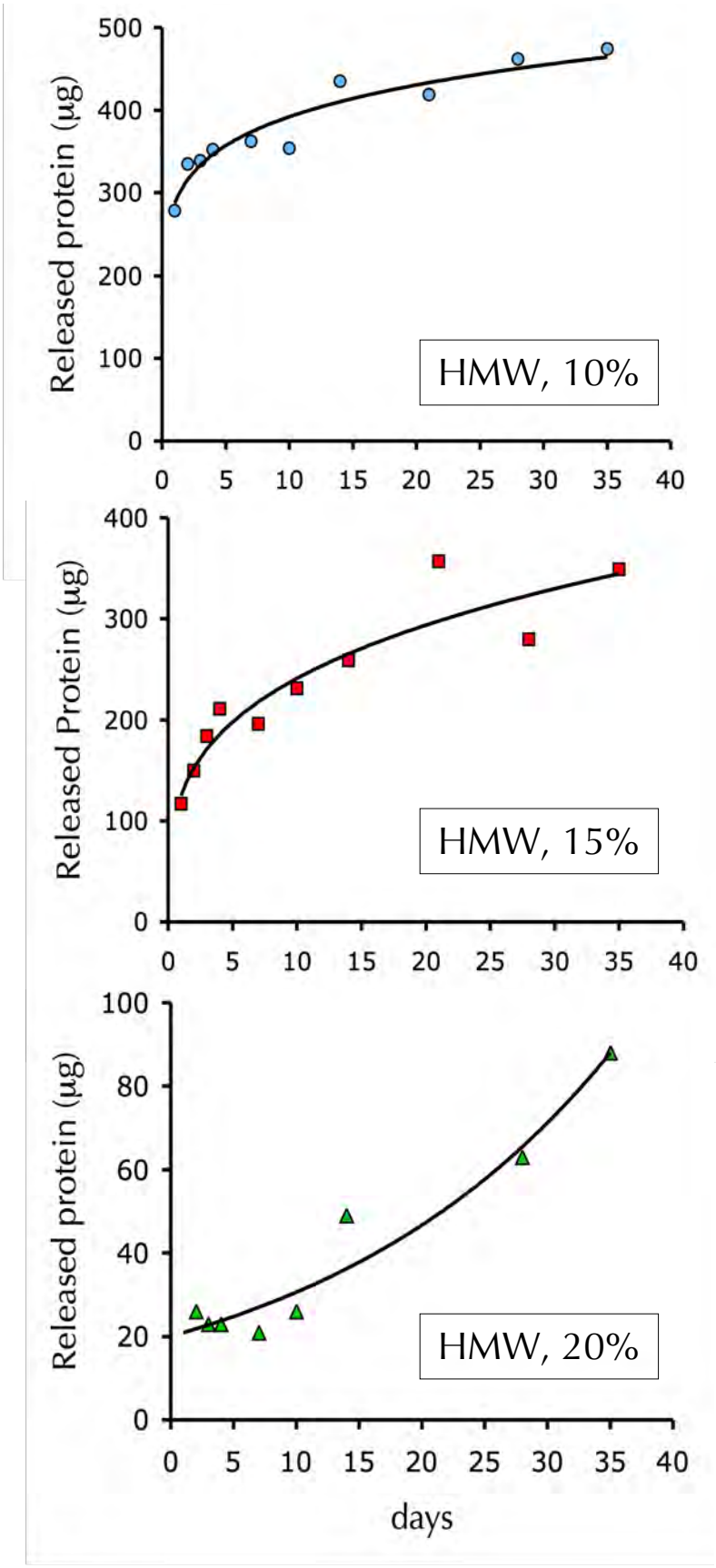

Figure 5. Protein release kinetics of HMW PLGA microspheres loaded with BSA. Protein release shows clearly different trends depending on the concentration of PLGA.

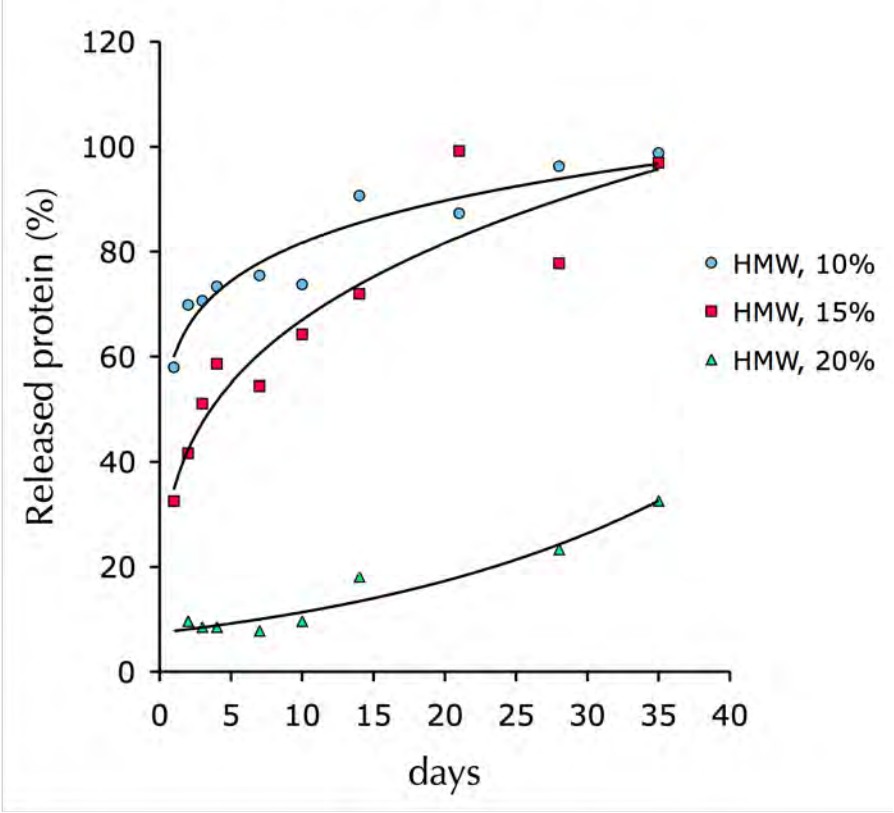

Figure 6. Protein release was normalized to the total amount of protein included in the testing microspheres. HMW, 10% shows a high burst rate of 60% during the first 24 hours followed by 80% cumulated protein release during the next 5 days. HMW 15% shows a burst rate of about 30% and gradually relases protein to a total of 80% in the next 15 days. Both 10 and 15% HMW PLGA release nearly 100% of encapsulated protein in 35 days. 20% HMW PLGA shows slow and sustained release of protein. Following the initial burst rate of about 8%, there is a lag period of about 10 days followed by slow release. A total of 35-40% of encapsulated protein is released during the first 35 days of incubation.

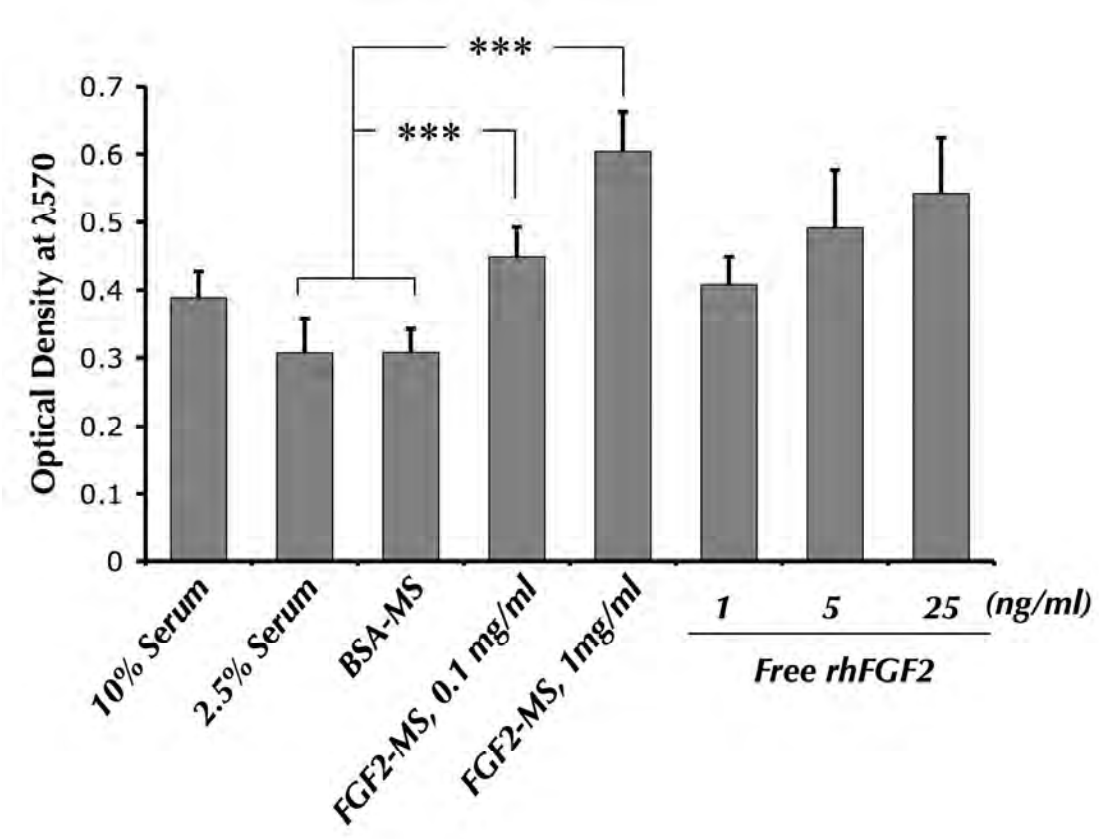

Figure 7. FGF2-MS bioactivity was determined on HUVEC survival survival using MTT assays. Cells were serum-deprived for 3 days in the presence of BSA-MS, FGF2-MS or free rhFGF2. FGF2-MS contains 10ng of FGF2 in each mg of MS.

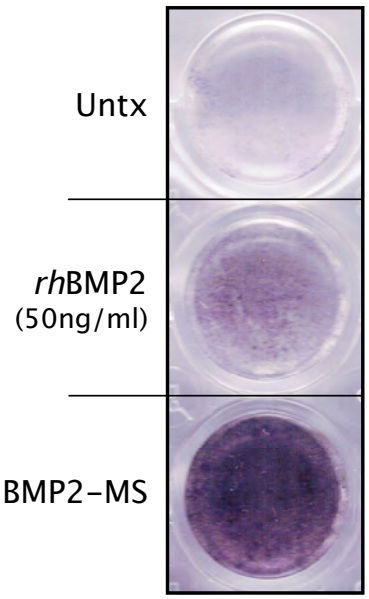

Fig 8. BMP2-MS bioactivity was determined for its ability to induce alkaline phosphatase activity in MC3T3 cell line. Cells were cultured in the presence of free rhBMP2 (50ng/ml) or BMP2-MS for 3 days and stained for alkaline phosphatase activity.

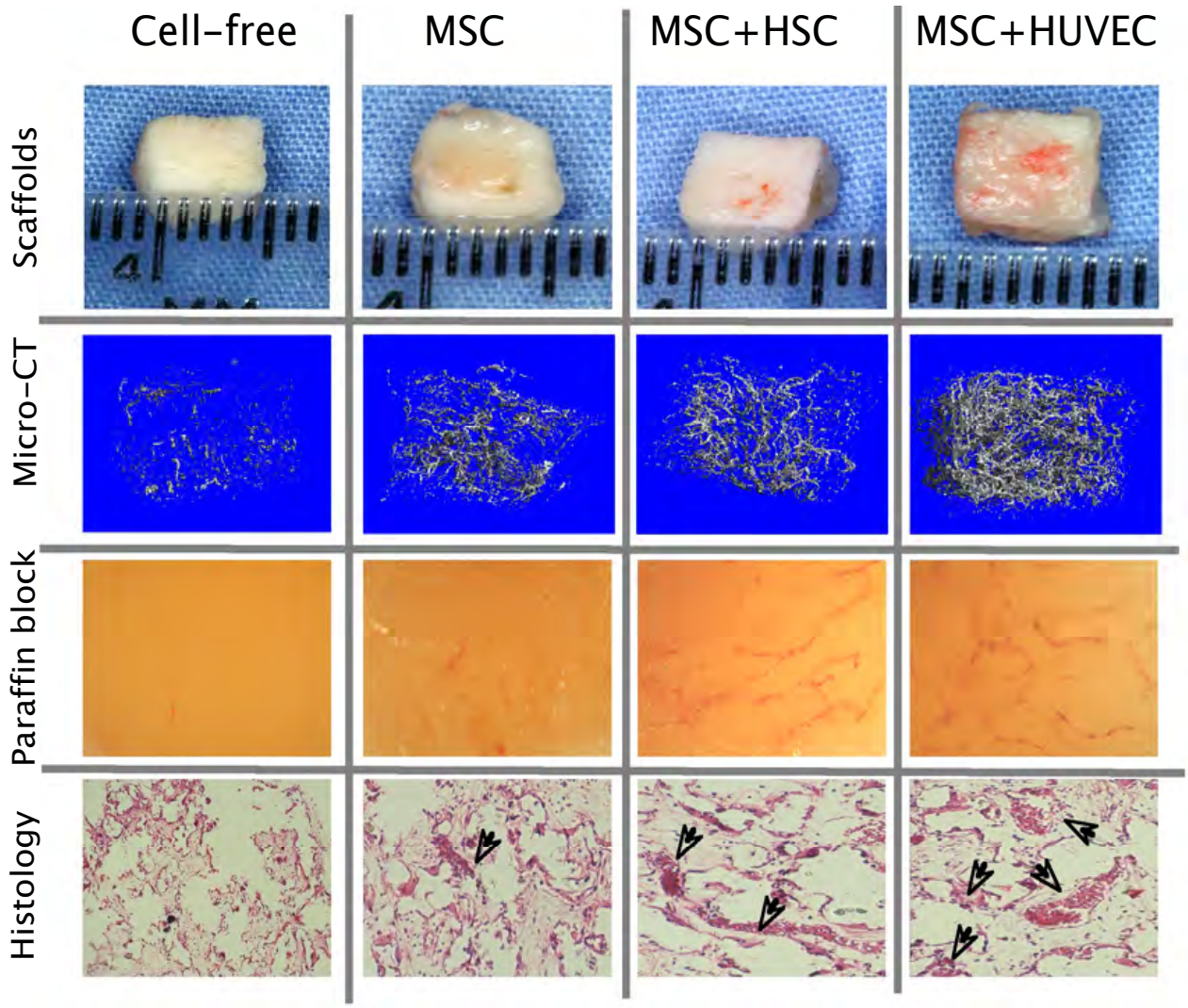

Figure 9: PCL-TCP scaffolds loaded with various combinations of cells (Cell-free, MSC alone, MSC+HSC and MSC+HUVEC) were perfused with Microfil at the end of experimental period and fixed in 10% formaline before taking photographs (**A**). The general shape was well preserved but the dimension of the construct was decreased by 33% after 8 weeks in vivo. The extent of Microfil perfusion was readily detectable by gross examination of the samples. The samples were then imaged by micro-CT (**B**). Quantitative analysis of the samples for the blood vessel volume showed that the constructs loaded with MSC+HUVEC cells showed most extensive blood vessel growth followed by those loaded with MSC+HSC and MSC alone. The scaffold implanted without cells showed minimal vascularization within the constructs. The constructs were further analyzed by histology which conformed extensive blood vessel formation in the MSC+HUVEC cell loaded constructs, confirming the visual and micro-CT data (**C**). The paraffin blocks of the constructs were prepared to assess the vascular formation through the length of the scaffold center (**D**). Note prominent neovascualrture in HUVEC/MSC.

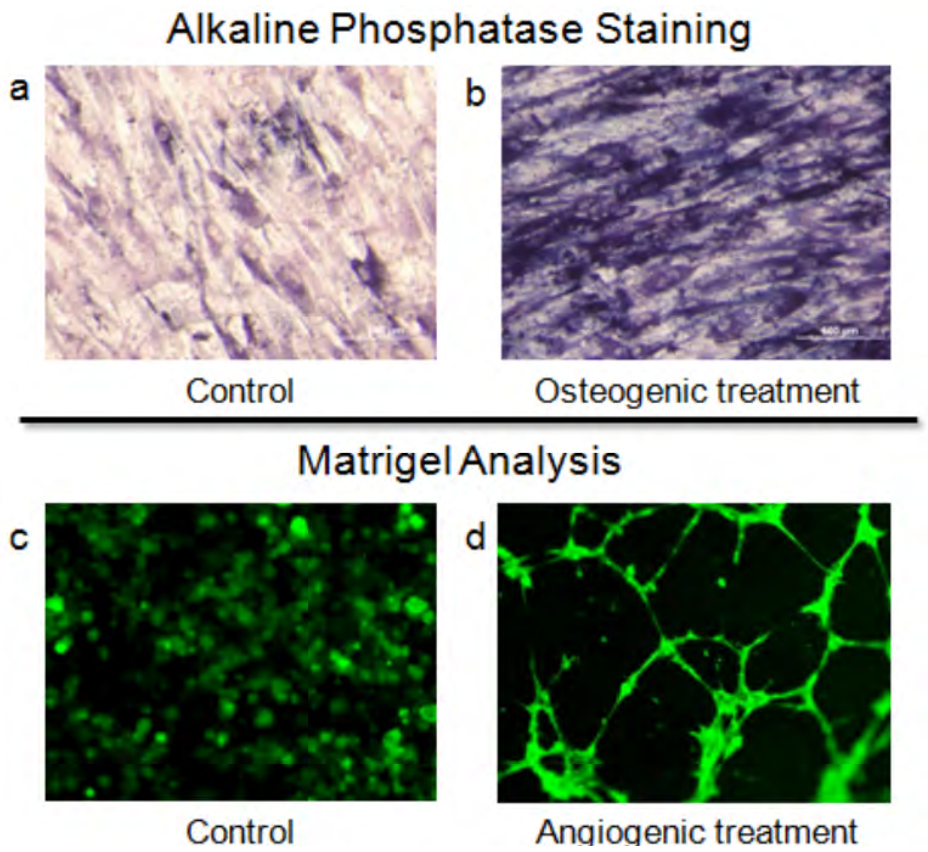

Figure 10. UCB-MSCs demonstrate multipotent capacity *in vitro*. The cells treated with FGF2 followed by BMP2 show high level of alkaline phosphatase activity (b) compared to untreated cells (a). UCB-MSCs treated with FGF2 and VEGF show capillary tubule formation on Matrigel (d). Untreated cells failed to form the tubules (c).

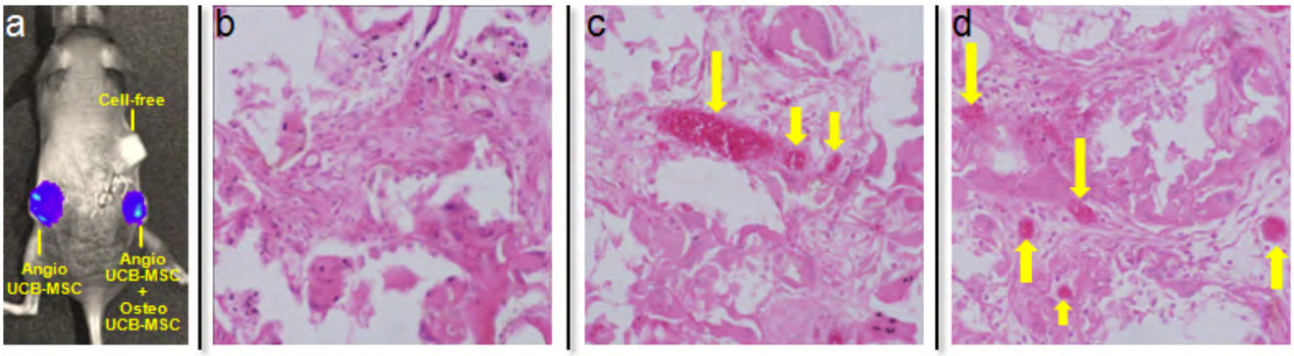

Figure 11. Histological analysis of implants. *In vivo* bioluminescence imaging (a) shows locations of implanted scaffolds and bioluminescent activity in both cell-containing constructs. Histological analysis shows no evidence of vessel formation in the cell-free scaffolds (b). Scaffolds seeded with angiogenic UCB-MSCs (c) and scaffolds seeded with both angiogenic and osteogenic UCB-MSCs (d) demonstrate histological evidence of microvasculature containing red blood cells

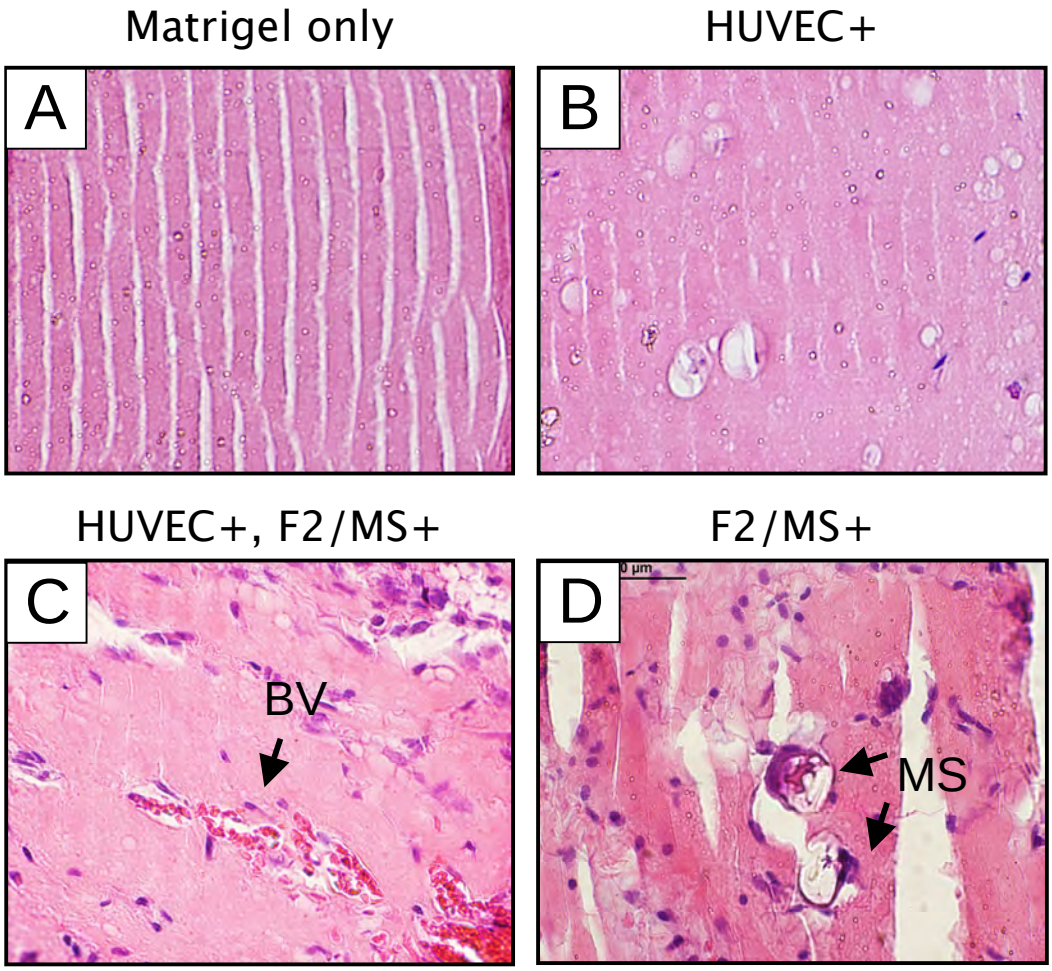

Figure 12. The *in vivo* effect of FGF2-MS on angiogenic cell survival and differentiation. 200 μl matrigel containing HUVEC only, FGF2-MS only, or HUVEC plus FGF2-MS, was subcutaneously injected. The matrigel scaffold was harvested in 10 days and examined by histology. The gel only group contained no cells (**A**). The HUVEC only group showed only a small number of surviving cells scattered within the gel (**B**). The group with both HUVEC and FGF2-MS showed numerous cells not only surviving but also forming blood vessels (**C**). The FGF2-MS only group also showed presence of cells in vicinity of microspheres (**D**).